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Report Prepared by: J. Howard Mueller
and
Pauline A. Miller

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PRINCIPAL INVESTIGATOR: J. Howard Mueller

Assistant: Pauline A. Miller

TITLE OF PROJECT: Production of Tetanus Toxoid

Objectives: To improve present yields and to study factors concerned.

ABSTRACT OF RESULTS - "Production of Tetanus Toxoid"

a. Since start of project:

Studies on growth requirements of C. tetani, and on factors concerned in the production of its toxin have been going on in our laboratory for 13 years. The original choice of the strain was arbitrary, since no basis was available for a more intelligent selection. Traditionally, tetanus cultures for toxin production had been maintained as spore suspensions. It was generally believed that success in obtaining toxin depended upon this procedure. Since the investigation of growth requirements demanded an almost daily actively growing seed culture the spore inoculum was not adopted, and daily transplants in glucose broth were used. This plan has been followed throughout the work. It resulted over the years in a culture which is atypical but constant in morphology, and does not readily sporulate. In certain nutritional environments it produces extraordinarily high titers of toxin.

The initial studies on growth requirements extended over 2 or 3 years(1). These requirements were complex and included at least one substance, folic acid, which was recognized and named during that time but was not isolated or available in pure form until later. When we undertook to apply our information on growth requirements to the production of toxin, modest success was achieved. A medium free from "peptones", and adapted to large scale production gave yields of toxin on the borderline of acceptability under National Institute of Health Standards(2). Attempts to improve yields by variation of nutritional composition within the known framework of growth requirements failed, and eventually were abandoned, and an opposite approach was undertaken. The plan was to select a complex, peptone-containing medium upon which more powerful toxin could be obtained and to attempt to characterise the essential chemical components of this medium.

For this purpose, the medium which was being used for tetanus toxin production at the Connought Laboratories in Toronto seemed suitable. It was prepared from autolysed hog stomach, and through the courtesy of Dr. Edith Taylor we learned the details of its preparation and use. Yields of toxin obtained with it, and with the Connought strain of the tetanus bacillus, were 2 or 3 times those obtained in our laboratory with the peptone-free medium. However, when our own strain was tried on the hog-stomach medium, toxin of several times this potency resulted. It was at first assumed, - on insufficient evidence, - that the peptic type of digestion occurring during the autolysis of hog stomach was an essential feature of the medium. Later, it was learned that a pancreatic digest of casein could replace the hog-stomach and offered certain obvious advantages over the latter. Digests prepared in our own laboratory, as well as various commercial preparations have been satisfactory, although variable. Through the courtesy of the Sheffield Farms Company, we have had adequate amounts of suitable lots of their preparation, "N-Z-Case", for chemical investigation.

The medium is composed of casein digest, beef heart infusion, glucose, additional vitamins, cystine and tyrosine, and inorganic salts. It is now rather generally employed, together with our strain of the tetanus bacillus, by manufacturers of pharmaceuticals in this country and many European laboratories. (3)

ABSTRACT OF RESULTS (cont.) - "Production of Tetanus Toxoid"

After lengthy and generally unprofitable exploration of a variety of methods of fractionation, it finally proved possible to separate the digest by means of reversible resins into acid, basic and neutral fractions which, when combined and added to culture medium yielded satisfactory titers of toxin. Omission of any of the three, or substitution of the products of acid hydrolysis of any of the three for the corresponding original fraction resulted in failure of toxin formation.

The basic fraction, making up about 20% of the total solids of the digest, seemed the simplest one with which to attempt further separation. Free histidine, arginine and lysine, as well as peptides of each, were present. Silver precipitation by conventional methods effected a reasonably satisfactory separation. The arginine and lysine fractions were respectively replaceable by pure free amino acids but free histidine did not replace the corresponding fraction from the digest. The latter, by paper chromatography, revealed the presence of several peptides containing histidine. These were separated to a considerable degree on Dowex 50 columns by the method of Hirs, Moore and Stein. After evaporation and removal of ammonium acetate buffer by sublimation the residual peptides were tested for toxin production by adding to an otherwise complete medium. It could be shown that each of the histidine-containing fractions, when added in suitable amount was capable of supplementing satisfactorily the deficient medium and that a powerful toxin was obtained. The most obvious explanation for this phenomenon seemed to be that histidine in peptide union was for some reason essential to the toxin-forming function of the tetanus bacillus. Since the various peptide fractions differed widely as to the other constituents there was no evidence for a specific linkage. Through the courtesy of Dr. du Vigneaud it was possible to test a few synthetic histidine dipeptides and to find that each of these in suitable quantity was as effective as the naturally occurring materials.

b. During current report period:

(1) Efforts to improve and regularize toxin yields on basic medium, success with which should be directly applicable to large scale production.

The frustrating element in all our work with tetanus has been the day-to-day (or month-to-month) variation in yields of toxin on the basic medium. Two tubes of this medium are included in each experiment as "positive controls". Too often the toxin titer of these tubes fails to come up to expectation and consequently the interpretation of the rest of the experiment is impossible. It has always been necessary, because of this fact, to repeat several times every experiment which seems significant. The same variation in titers obtained in production laboratories still represents a major defect in the method. Many experiments have been carried out during the current year directed especially toward elimination of this variation. Some improvement seems to have been achieved but there still remain elusive variables, perhaps inherent in the tetanus strain itself and discouraging differences in yield still occur. Factors within the medium which have received particular scrutiny include the following (a) inhibitory materials present in the tryptic digest of casein, (b) quantity, type, and "condition" of the added Fe, (c) concentration and relative proportions of each component of the medium, (d) the tetanus strain and (e) technical errors.

ABSTRACT OF RESULTS (cont.) - "Production of Tetanus Toxoid"

(a) Tryptic digests of casein vary in efficacy when used in the medium. At least some of this variation seems due to the presence of materials inhibitory in some way to toxin formation. These have not been identified, but there is some evidence that traces of fatty acids or other lipoidal material is concerned. Empirically, we have employed charcoal adsorption to remove these substances, in spite of obvious objections to such a procedure. During the year we have replaced this method, with resulting improvement in toxin, by a method devised at the New York State Laboratories (4) which consists in forming a precipitate of calcium phosphate in a 20% solution of the tryptic digest at 100° and about pH 9-10, which is then filtered off. The precipitate evidently adsorbs the inhibitory material. The method, however, has the theoretical disadvantage that boiling a mixture of amino acids and peptides at pH10 seems pretty drastic, and the practical disadvantage that pH10 is not readily determined either by indicator or glass electrode, so that the procedure is difficult to reproduce exactly. In recent weeks we have adopted a third device based on casual observations over a long period. This consists in forming in the cold a rather heavy precipitate of $BaSO_4$ in the tryptic digest solution, and centrifuging it off. A 10% solution of digest is mixed with half its volume of cold saturated $Ba(OH)_2$ and immediately neutralized with about 0.5 N H_2SO_4. The precipitate, usually colloidal at the neutral point, is caused to "break" by the careful addition of strong H_2SO_4 a drop at a time, and then centrifuged perfectly clear.

All three of these methods would probably remove fatty acids. The charcoal method probably removes significant quantities of essential components, and perhaps fails to remove all inhibitory materials at "safe" levels. Both the calcium phosphate and the $BaSO_4$ method permit increasing the concentration of the resulting digest in the medium from 1.5% (giving optimal results in the charcoal method) to 2.25%, with increased yields of toxin, - (from 80-100 Lf with charcoal treated NZC to 120-130 Lf and occasionally even up to 160 Lf).

(b) Fe. Mercks "Iron by Hydrogen" a very finely divided black powder, about 3 mg per 20 ml of medium gives optimal results. Other brands of reduced iron have invariably been inferior. Spectroscopic examinations and many experiments with other trace metals have provided no clue to the difference. The addition of exactly 3 mg of this powder to each of a series of 20-30 tubes of medium making up an experiment poses a technical problem which we have not thus far solved. The difficulty is increased when we employ 5 ml of medium instead of 20 ml in order to conserve valuable fractions of tryptic digest. The required 0.75 mg of Fe simply cannot be weighed and transferred with precision. Soluble iron salts are not satisfactory. A good deal of time, during the year, has been devoted to attempts to prepare Fe-containing tablets. Colleagues at the Massachusetts College of Pharmacy have been most helpful. As "fillers" we have tried lactose, glucose, gelatine, celite and aluminum silicate in various combinations. None of these have been as effective as the pure iron powder. Of course this technical difficulty does not exist in large-scale work where considerably larger amounts of Fe may be weighed and transferred with negligible error.

ABSTRACT OF RESULTS(cont.) - "Production of Tetanus Toxoid

(c) Concentrations and proportions of various ingredients of medium. The increase of concentration of tryptic digest made possible by the Ca or the Ba treatment made it necessary to re-examine the concentration of all other components to make certain that none became limiting. Slight increases in glucose, K and Mg seemed indicated and some decrease was made in NaCl and $Na_2H\ PO_4$ in order to keep the osmotic pressure of the medium within reason. A curious relationship between cystine and uracil emerged for which we have no explanation, nor even as yet a perfectly clear understanding. Omission of uracil results in poor growth and little toxin. Addition of increasing quantities in a series of tubes results in toxin titers rising along a reasonably regular curve and then falling somewhat as more uracil is added. The amount giving the best result seems to vary with the concentration of cystine. However, if really excessive quantities of uracil are added the effect of cystine is negligible and it may even be omitted entirely, but toxin titers, though good, have never reached levels obtained with smaller amounts of uracil and careful adjustment of cystine. This type of quantitative relationship may exist between other components of the medium although many experiments designed to bring it to light have failed to do so.

(d) The strain of C. tetani is unstable and our methods of carrying it are probably not optimal. Considerable effort has gone into attempts to improve this routine, thus far with no notable progress. We maintain a stock of dry-ice frozen cultures for emergency use when the passage culture appears at fault, and the New York State Laboratories maintain a stock of lyophylized tubes of the strain. It would be useless to detail the various expedients we have tried, but we simply point out that the matter has received and will continue to receive experimental attention.

(e) Technical errors in setting up these complicated experiments seem impossible to avoid. The basic medium is prepared from about 15 stock solutions. We attempt to make each of these correctly in the first place and in quantities adequate for a considerable period of time, and to replace each with a new solution which is checked against the old one before it is entirely exhausted. Every experiment (usually one a day) consisting of 10 to 30 tubes of medium, each varying from the others in concentration of one or more ingredients, implies an hour or somewhat more of extremely careful pipetting. A few interruptions during this time can thoroughly wreck the experiment. If we become aware of an error, of course, it is rectified even if it means throwing out the whole business and starting over. If we do not detect it, the experiment is completed, eventually inoculated, held in the water bath for five days and flocculations carried out to measure toxin yields. At this point if the results are low, irregular or entirely absurd, we can only ask ourselves (1) did we make a mistake (2) was a solution wrong (3) has the toxigenicity of the strain fallen off or (4) - at this point there is an inclination to invoke the metaphysical.

Obviously we make every effort to avoid mistakes in pipetting and measuring. As often as it is practicable we work together at this part of the experiment, one pipetting and one checking. As the experiments become more complex and of the type to be considered in the next part of this report, in which tryptic digest, instead of being added as a single solution is replaced

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by a series of amino acids and peptide fractions, the difficulty increases. Each experiment is repeated several times and only when a series of entirely consistent results are obtained do we feel safe in concluding that the point under investigation has been established.

(2) Further efforts to characterize the acid-labile components of tryptic digest of casein essential in toxin formation.

For convenience, let us assume that the tryptic digest has been separated by means of reversible resins into an acidic fraction (A), a basic fraction (B) and a neutral fraction (C). This has been accomplished batch-wise by methods devised two or three years ago involving two resins and three steps, and by means of a fraction-collector and a single resin during the current year. The latter method is certainly more convenient and probably superior to the former. Details do not seem pertinent to this summary but of course are available to anyone interested.

Fraction B, as stated in the introduction, seems to be fully elucidated. It is adequately replaced in the presence of A and N by arginine, lysine and glycyl-histidine, or by several other peptides of histidine. We at first undertook to compare quantitively the effect of several histidine peptides and devoted a great deal of time to the preparation of a series of these compounds, since they were nowhere available. Much of this effort was wasted through our inexperience with this type of organic preparation and through unfortunate choice of methods. Later, with the generous cooperation of Drs. Max Bovarnick (formerly of this department) and Ernest Borek we obtained fair yields of three such peptides in their laboratories at the Brooklyn Veterans Administration Hospital. Still later we prepared a really adequate amount of glycyl-histidine via chloroacetyl-histidine and NH_4OH, the general method used by Greenstein (5) for other glycyl-peptides, though not used by him for the histidine derivative. We found that the chloroacetyl intermediate could readily be purified and crystallised by fractionation of the crude reaction mixture on a resin column, and that the final peptide could be similarly separated from by-products after the NH_4OH treatment. We therefore now have adequate amounts of this peptide for many experiments and a method making readily possible the preparation of additional amounts should need arise.

We have decided, however, to defer quantitative comparison of a variety of histidine peptides until it is possible to use more reproducible, and, if possible, synthetic materials for the A and the N fractions. For the moment, therefore, it appears that our present knowledge of the B fraction is sufficient for our immediate purposes.

Fraction A makes up roughly 20% of the tryptic digest. It owes its acidic nature for the most part to the fact that it contains all of the phosphorus of the casein (a phosphoprotein). Only a small portion of this phosphorus is inorganic, the bulk of it being combined in a relatively complex peptide structure containing (at least) serine, glutamic acid and isoleucine, and probably also a number of other amino acids. By means of anion exchange

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resins and the fraction collector we have convinced ourselves that toxigenicity and bound phosphorus are reasonably parallel and inseparable by that means. We are fairly certain that the phosphorus itself is not the active material because certain fractions of a tryptic digest of beef-heart, containing no phosphorus, carry "A" activity. These fractions have not yet been studied further. It is possible that it is the peptide-bound serine in the A fraction of casein which is the essential component. We have synthesized glycyl-L serine by Greensteins method and found that it will not replace the A fraction. It remains to try other types of serine peptide and to explore the possibility of some other type of peptide alone or in combination with serine. Again, we plan to defer this work until a better N fraction substitute becomes available.

Fraction N represents the largest fraction of the tryptic digest (about 60%) and the most complex. It contains most of the "neutral" amino acids in free form and large quantities of peptides of varying degrees of complexity. From early experiments on growth we know that of these amino acids, valine, leucine, isoleucine, methionine, serine, threonine, phenylalanine and tryptophane are either essential or stimulatory to growth. Only in the case of the last two have we been able to demonstrate a direct relation to toxin formation and to determine the optimal concentrations. For the others this has not yet been possible, perhaps because each occurs in such a variety of peptides, as well as in the free form, that they are represented in every sub-fraction of the N, no matter how obtained. We have been able to show that each (and all) of them can be added in the form of L synthetic amino acids along with the N fraction and that in modest concentration no material inhibition of toxin results. This makes it possible to subdivide N in a variety of ways and to test the fractions for toxin formation in a basic medium containing all of these amino acids and hence not accidentally deficient in one or more simple known compounds.

Pursuing this general plan we have separated N into a series of fractions by applying it to an anion exchange resin column and eluting it by the displacement procedure of Partridge (6). Testing such fractions for toxin formation shows activity more or less throughout all of them but more pronounced in some areas than in others. This suggests the same type of effect found in the B fraction where each peptide containing histidine was effective and may mean activity with several peptides each containing a certain amino acid. It does not exclude the possibility that concomitant presence of two kinds of peptide are essential. It also does not exclude the possibility that the essential materials are not peptide in nature at all.

Our next step was to select a series of adjoining fractions showing the greatest effect on toxin formation, and to re-fractionate them through a cation exchange resin, again by the displacement procedure. The resulting fractions again showed a well defined peak of activity, but paper chromatography readily demonstrated the presence of such a variety of peptides that there was no possibility of a clue as to involvement of any particular amino acid component. Our next step was to undertake a better separation by means of the Hirs, Stein and Moore elution method (7) using the resin Dowex 50.

ABATRACT OF RESULTS (cont.) - "Production of Tetanus Toxoid"

Somewhat improved separation resulted, but the active material remained complex on paper-chromatography.

We have now further separated this active fraction by adsorption on and elution from charcoal columns, separating certain inert materials, but still recovering an active fraction showing several components by paper chromatography. These various steps have eliminated sufficient inert solid to make an actual fractionation of the remaining active material feasible on filter paper sheets. Past experience with this method has not been altogether encouraging, since (1) extracts of the filter paper itself seem to inhibit toxin formation and (2) it does not always seem possible to wash the adsorbed material off the paper. However, by using previously washed and treated paper, and extraction with 0.01 N H_2SO_4 which can readily be removed with Ba $(OH)_2$, we have had a few encouraging experiments. It appears that the active fraction moves rapidly on paper, using secondary butanol-formic acid as the solvent. The experiments are now at this point. For reasons to which we have alluded above, they will have to be repeated several times. If all goes well, it may then be possible, by paper chromatography, to obtain fairly definite information as to the composition of the active fraction, and to attempt somewhat more intelligently a substitution of known peptides or other compounds for the naturally-occurring material.

This aspect has already received a reasonable amount of empirical attention. We have tried a variety of peptides -- some begged, others bought, and others prepared by us. None have been effective, but the possibilities have not been covered. A great deal remains to be done, and if the analytic experiments provide a clue, much effort may be saved.

References:

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(2) Mueller, J. H. and Miller, P. A. 1943. large-scale production of tetanal toxin on a peptone-free medium. J. Bact., 47, 15
(3) Mueller, J. H. and Miller, P. A. 1954 Variable factors influencing the production of tetanus toxin. J. Bact. In press
(4) Hendry, J. L. and Wheeler, M. W. 1954 In preparation.
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(6) Partridge, S. M. and Brimley, R. C. 1952 Displacement chromatography on synthetic ion-exchange resins. 8. A systematic method for the separation of amino acids. Biochem. J. 51, 628
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PLANS FOR FUTURE: - "Production of Tetanus Toxoid"

Immediate and Long Range: Work is at present continuing on the neutral fraction, and on efforts to further improve the basic formula for toxin production. Our ultimate aim is the identification of the remaining unknown constituents in the tryptic digest and in the heart infusion.

PUBLICATIONS

(1) Variable factors influencing the production of tetanus toxin. J. Howard Mueller and Pauline A. Miller.
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(2) Muscle extractives in the production of tetanus toxin. Nusret H. Fisek, J. Howard Mueller, and Pauline A. Miller
Journal of Bacteriology. 1954 In press.

(3) Participation of peptides in tetanus toxin production. J. Howard Mueller and Pauline A. Miller.
Transactions of the New York Academy of Sciences. 1954 In press.